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14. ABSTRACT

The purpose of this proposal is to determine the effects of stromal TGF-beta signaling inhibition by SD-208 (a TbetaRI-specific inhibitor) on prostate cancer growth, by using both in vitro prostate cancer/stromal cell co-culture model and in vivo xenograft model. In this initial funding year, we have tested the SD-208 efficiency in inhibiting TGF-beta signaling in prostate stromal cells. We further demonstrated that TGF-beta signaling in prostate stromal cells induced a complex response in prostate cancer cells. This response can be inhibited by either knockout of TbetaRII from mouse prostate stromal cells or by addition of SD-208 into the co-culture of human prostate cancer cells and human prostate stromal cells. We are using cDNA microarray and qPCR to identify the potential signaling molecules regulating these events. Based on these in vitro results, we are now carrying out the in vivo studies. We expect to finish all these studies at the conclusion of this award and present these sets of data in the final report.

15. SUBJECT TERMS
Prostate cancer; transforming growth factor beta; SD-208, a transforming growth factor beta receptor I specific inhibitor

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W81XWH-07-1-0200 "Attenuated Transforming Growth Factor Beta Signaling as a Therapeutic for Prostate Cancer Progression"

Introduction:

Our studies have defined reactive stroma in human prostate cancer progression (1). TGF-β is overexpressed in most carcinomas associated with a reactive stroma, including prostate (2). TGF-β1 stimulates expression of angiogenic growth factors from stromal cells, such as VEGF, FGF-2, and CTGF (3, 4). Subcutaneous injection of TGF-β1 was sufficient to induce a stromal reaction with differentiation to myofibroblasts, enhanced collagen production and stimulated angiogenesis (5, 6). Therefore, we believe that TGF-β1 is a key factor inducing a reactive stroma in wound repair and cancer. We have reported that TGF-β1 induces human prostate fibroblasts to differentiate to myofibroblasts and express markers of reactive stroma *in vitro* (1). By using the differential reactive stroma (DRS) xenograft model developed in this lab (7-9), we showed that human prostate stromal cells are differentially promoting rate of cancer progression. Microarray analyses showed that tumor promoting prostate stromal cells express several TGF-β regulated genes including CTGF (9). Of interest, a major role of tumor-promoting stromal cells is rapid initiation of angiogenesis in the xenograft tumors (1). The pro-angiogenesis and tumor-promoting properties of stromal cells is blocked by inclusion of neutralizing antibodies to TGF-β or latency associated protein (LAP) in the xenograft (8). SD-208 is a specific inhibitor of TβRI kinase activity (10-12). We proposed to test whether SD-208 inhibition of TGF-β signaling will modulate prostate cancer reactive stroma and affect prostate cancer progression.

Body:

Task 1 will test the efficacy of SD-208 in the human DRS xenograft model.

We proposed in this **Task** to 1, test the efficacy of SD-208 in inhibition of TGF-β induced biology in human prostate stromal cells *in vitro*; 2, test the efficacy of SD-208 in inhibition of human prostate cancer progression in DRS xenograft model *in vivo*, such as differences in tumor mass between the SD-208 treated group and the control group; 3, analyze the histopathology of the DRS tumors, including reactive stromal markers and angiogenesis.

As disclosed in our previous communications with PCRP of DOD CDMRP, we encountered significant difficulty in acquiring sufficient amount of SD-208 compound from *Scios, Inc*. to carry out the proposed experiments in our original proposal, due to a sudden closure of *Scios, Inc*. by its parent company (*Johnson and Johnson*). The TGF-β inhibitor research group of *Scios, Inc.* was relocated to *Alza Corporation*, which is "a member of the *Johnson & Johnson* Family of Companies, serves as one of *Johnson & Johnson*'s Pharmaceutical Research & Development sites" (reference to http://www.alza.com/alza/). We were informed that SD-208 manufacturing was discontinued for the time-being, and there was no plan in place to resume its production in the very near future, which meant that there was limited amount of SD-208 available from the company.

After working closely with the newly assigned company, *Alza Corporation*, we finally received a shipment of 3.0 gram of SD-208 compound, which is the maximum amount we could get from the company. We realized that the limitation of SD-208 compound shipped to us might limit the scope of the works proposed in our original proposal, especially in **Task** 2, which required much more SD-208 compound than what was available to us. Therefore, we had re-designed **Task** 2. The revised **Task** 2

will study the effects of stromal TGF-β signaling inhibition by SD-208 on prostate cancer cell proliferation/morphology using an *in vitro* co-culture model of co-culturing human prostate cancer (LNCaP) cells with human prostate stromal cells as described below. Since this revised **Task** 2 requires much less SD-208, we will be able to finish this **Task** with the limited amount of SD-208 available to us. Our revised SOW was approved by PCRP on Oct 11, 2007.

The limited amount of SD-208 allows us to perform the *in vivo* experiments in **Task** 1. However, all the experiments in **Task** 1 have to be done in an "error-free" manner since we do not have extra SD-208 compounds to repeat these *in vivo* experiments more times than necessary. In contrast, all the experiments in **Task** 2 require much less SD-208. Therefore, instead of simultaneously carrying out all the experiments in **Task** 1 and **Task** 2, we have used a conserved strategy to first carry out the *in vitro* experiments in **Task** 2 and part of **Task** 1. These *in vitro* experiments together will demonstrate SD-208 effects on inhibition of TGF-β signaling in prostate stromal cells and on human prostate cancer (LNCaP) cell proliferation / morphology in the LNCaP-stromal cell co-cultures *in vitro*. Since we have demonstrated an SD-208 induced biology *in vitro* (as described below), we are beginning the *in vivo* studies and will finish these studies by the end of this award. The results from these *in vivo* studies will be presented in our final report.

In **Task** 1, we have tested the efficiency of SD-208 compound in inhibiting TGF-β signaling in human prostate stromal cells, such as HPS-19I cells (13). $(CAGA)_{12}$MLP contains 12 copies of CAGA Smad binding elements driving luciferase expression. It is commonly used for testing TGF-β response in cells *in vitro* (14). As shown in Figure 1, $(CAGA)_{12}$MLP transfected HPS-19I cells expressed minimal luciferase activity. 50 μM of TGF-β induces a 24-fold increase in $(CAGA)_{12}$MLP promoter activity and this induction is inhibited by SD-208 in a dosage-dependent manner. The TGF-β induced $(CAGA)_{12}$MLP promoter activity is restricted to less than 2-fold by 100 nM of SD-208. Furthermore, the induction is reduced to basal level by 400 nM and 800 nM of SD-208. Therefore, we demonstrate that SD-208 is able to inhibit TGF-β signaling in human prostate stromal cells *in vitro*. We chose 400 nM of SD-208 for our *in vitro* co-culture experiments in **Task** 2. This result, together with all the results from **Task** 2, will set up the foundation for the *in vivo* experiments in **Task** 1, which are expected to be finished at the conclusion of this award.

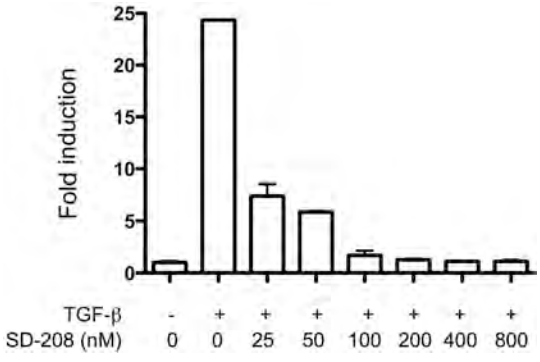

Fig 1. SD-208 inhibition of TGF-β signaling in human prostate stromal cells.

Revised Task 2. Our revised **Task** 2 will test the effects of inhibition of TGF-β signaling in prostate stromal cells by SD-208 on prostate cancer cell growth and/or morphology changes *in vitro* in the cancer / stromal cell co-culture system.

In the revised **Task** 2**,** we propose to co-culture human prostate cancer LNCaP cells with mouse and human prostate stromal cells in 1% FBS in the presence of SD-208 or control. LNCaP prostate cancer cell proliferation (growth) and morphology (potential related to metastasis potential) will be assayed. Co-cultured LNCaP prostate cancer cells will be labeled with GFP and human prostate stromal cells will be labeled with "CellTracker Red CMTPX" from Molecular Probes™ in 1% FBS in the presence of SD-208 or control, followed with cell sorting for green fluorescence LNCaP

cells and Red fluorescence stromal cells. Finally, total RNA will be extracted from the above cells and cDNA microarray and real-time PCR will be performed to detect and verify the molecular changes in LNCaP cells and stromal cells when stromal TGF-β signaling is inhibited by SD-208 in the co-culture.

Co-culture of LNCaP cells with mouse prostate stromal cells

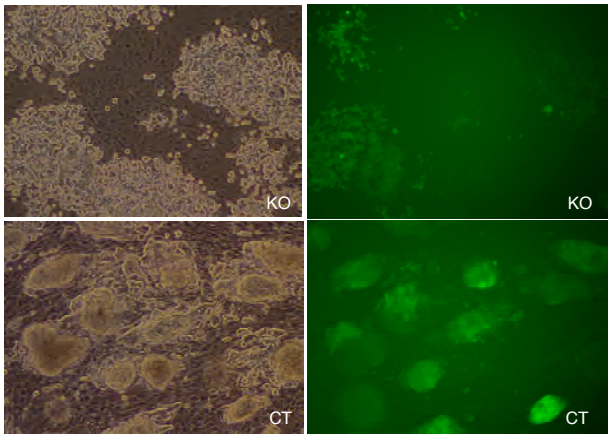

Fig 2, LNCaP cells co-cultured with mouse prostate stromal cells. KO: TβRII knokcout, CT: TβRII control.

We first set up *in vitro* co-cultures in which LNCaP cells were co-cultured in 1% FBS with mouse prostate stromal cells with or without intact *TβRII* (TβRIICT (control) cells *v.s.* TβRIIKO (knockout) cells as described in (15). Note that the LNCaP-GFP$^+$ cells used in these sets of experiments were kindly provided by Dr. Roy-Burman (16) and they are not the same LNCaP-GFP$^+$ cells that we have recently generated as described below. Our preliminary data showed that LNCaP cells grew differently and exhibited different morphology as shown in Figure. 2. This result indicates a profound effect of disruption of TGF-β signaling in prostate stromal cells on prostate cancer cell growth.

To reveal the molecular mechanism that regulates this phenomenon, we extracted total RNA from these cultures and carried out a human cDNA microarray to identify differential gene expression in LNCaP cells co-cultured with mouse prostate stromal cells with or without intact TGF-β signaling. Interestingly, one of the up-regulated genes in LNCaP cells co-cultured with TβRIIKO stromal cells is PAcP, the cytoplasmic form of which has been previously shown to be able to dephosphorylate HER-2 *etc*, therefore, regulate prostate cancer cell proliferation and behavior (17). As shown in Figure 3, our RT-PCR result confirmed an up-regulation of PAcP in these LNCaP cells using primers designed specific for human PAcP. We will further confirm this data with qPCR. These data shed light on the potential involvement of EGF signaling pathways in regulating LNCaP cell growth in these co-cultures.

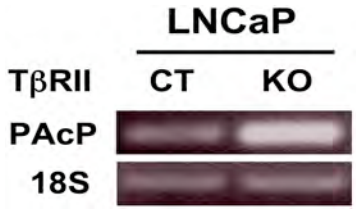

Fig 3, Stromal TGF-β signaling inhibits PAcP expression in LNCaP cells.

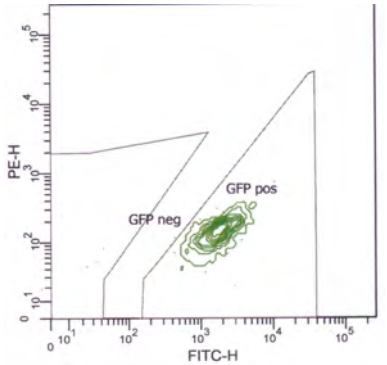

Fig 4. Over 99% of LNCaP cells are GFP$^+$ after two rounds of cell sorting.

Co-culture of LNCaP cells with human prostate stromal cells

To establish an LNCaP cell line that universally expresses GFP at early passage, we packaged amphotropic retroviruses by transfection of pBMN-I-EGFP vector into amphotropic phoenix retrovirus packaging cells and used these viruses to infect LNCaP cell at early passage following protocols described previously (9, 15). To enrich the LNCaP-GFP$^+$ cells, we ran two-rounds of green fluorescence-based cell sorting. As shown in Figure 4, after the second round of cell sorting, LNCaP-GFP$^+$ cell population is enriched to greater than 99% of total LNCaP cell population. Therefore, we have generated a "pure" LNCaP-

GFP$^+$ cell population for the proposed co-culture experiments. Similarly, we have also generated an LNCaP cell line overexpressing HA tagged constitutive active TGF-β1.

We have optimized LNCaP/human stromal cell co-culture conditions. We carried out the co-culture in PRMI 1640 with 0.2% FBS in the presence/absence of 50 pM TGF-β with/without 400 nM SD-208 treatment. As shown in Figure 5, after 15-day of co-culture, LNCaP cells in the LNCaP/HPS-19I co-culture maintain mostly flat. However, the LNCaP cells grow into three-dimensional structures when exposed to TGF-β. The formation of these three-dimensional structures was blocked by 400 nM of SD-208. Therefore, we demonstrated a morphology change of LNCaP cells induced by TGF-β treated prostate stromal cells and this morphology change was blocked by TβRI-specific inhibitor, SD-208. Total RNA has been extracted from each of these co-cultures. We are sending these RNA

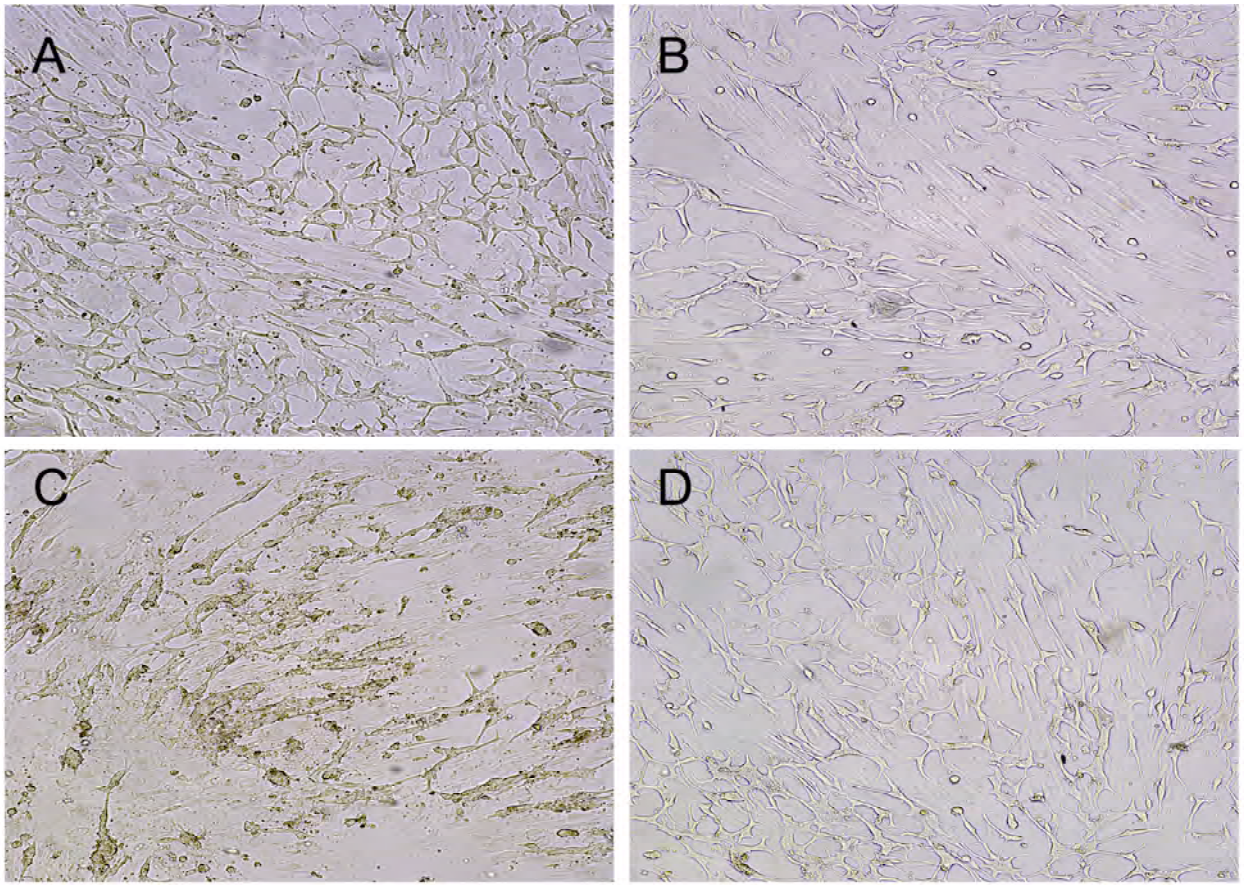

Fig 5. The morphology of LNCaP cells in co-culture with human prostate stromal cells under different treatments. Treatments for these co-cultures include A, Control; B, SD-208 alone; C, TGF-β1 alone; D, TGF-β1 and SD-208.

samples to a cDNA microarray core facility for a gene expression profiling analysis. We will compare the gene expression profile from these microarrays with the gene profile from our previous array to identify the common genes differentially expressed in LNCaP cells induced by TGF-β regulated stromal cells. We will use qPCR to confirm the expression patterns of genes of interest.

Due to the morphology differences presented in LNCaP cells under different co-culture conditions, it is difficult to directly quantify and compare the LNCaP cell numbers in these co-cultures. We tested different methods to quantify the LNCaP cell population in these co-cultures to compare LNCaP cell proliferation. We first tested using the flow Cytometry to quantify LNCaP cell population

by analyzing the percentage of GFP positive LNCaP cell population out of the total cell population (GFP positive LNCaP cells plus GFP negative prostate stromal cells). We did not detect significant change in the percentage of GFP positive (LNCaP) cells. However, we noticed a difficulty in preparing individual LNCaP cells for flow Cytometry analysis from the TGF-β treated co-cultures, due to the compact three-dimensional structure formed in this type of co-culture. Significant loss of LNCaP cell population was observed during the filtration step that removes the big cell aggregates before samples applied to cell sorting. Therefore, this analysis may underscore the LNCaP cell population in TGF-β treated co-cultures.

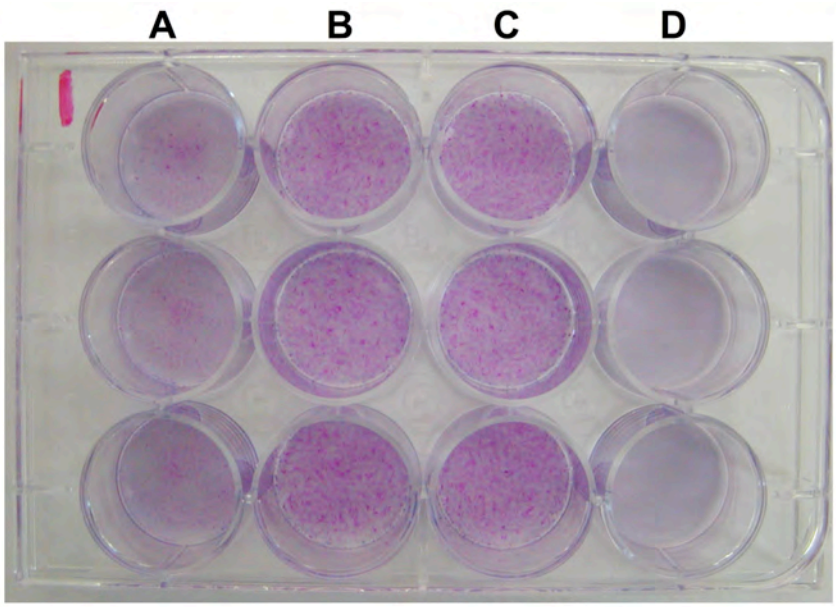

Fig 6. Rhodanile Blue staining of co-cultures, each condition in triplets. Samples include HPS-19I co-cultured with A. LNCaP; B. LNCaP overexpressing TGF-β; C. LNCaP treated with external TGF-β. D. HPS-19I alone.

Rhodanile Blue is a cellular dye preferring to stain epithelial-origin cells. A cell quantification method has been developed and used to quantify human prostate cancer cells in the co-culture of human prostate cancer cells and stromal cells (18). We tested using this method to quantify LNCaP cells in our co-culture system. As shown in Figure. 6, we observed an increase in Rhodanile Blue dye staining in the TGF-β treated co-cultures (*Fig 6**C***) and in LNCaP cells overexpressing constitutive active TGF-β ligand (*Fig 6**B***) compared to control co-cultures (*Fig 6**A***). We will repeat these experiments to confirm these observations. We may also try to use/develop other methods to quantify LNCaP cell proliferation in these co-cultures as alternative approaches.

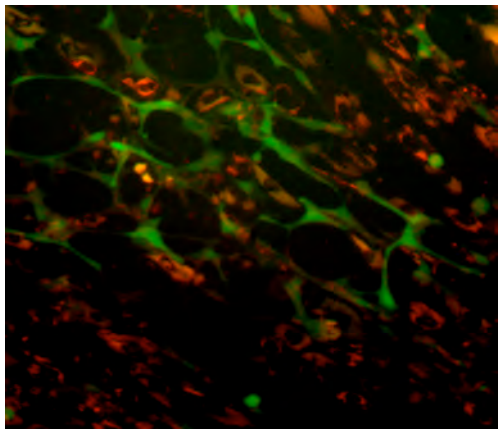

Fig 7. Co-culture of LNCaP-GFP+ cells with "CellTracker Red CMTPX" labeled HPS-19I cells.

Finally, we have optimized the experimental conditions to label human prostate stromal cells with "CellTracker Red CMTPX" from Molecular Probes™. By using this protocol, the labeled confluent human prostate stromal HPS-19I cells maintained their red fluorescence after extensive culture (>30 days). Figure 7 shows a typical micrograph of LNCaP-GFP^{+} cells co-cultured with HPS-19I cells labeled with "CellTracker Red CMTPX". However, co-fluorescent cells were observed, potentially due to the uptake of covalently labeled membrane proteins (peptides) from stromal cells by LNCaP cells. Therefore, the usage of "CellTracker Red

CMTPX" in labeling prostate stromal cells in the co-cultures may be limited.

Key Research Accomplishments:

- Acquisition of SD-208 compound for both *in vitro* and *in vivo* studies.
- Re-designation and modification of research **Tasks** based on the limited amount of SD-208 available to us.
- Human cDNA microarray on total RNAs from *in vitro* co-culture of human prostate cancer (LNCaP) cells and mouse prostate stromal cells with or without intact TGF-β signaling. This microarray identifies that stromal TGF-β signaling regulates the expression of PAcP in LNCaP cells. The cellular form of PAcP may play a role in regulating LNCaP cancer growth co-cultured with prostate stromal cell.
- Generation of dosage response curve of SD-208 in inhibiting TGF-β pathway in human prostate stromal cells.
- Generation and enrichment of GFP-labeled LNCaP cells (>99% positive for GFP) along with the LNCaP cells overexpressing constitutive active TGF-β.
- Optimization of culture conditions for *in vitro* co-culture of human prostate cancer cell (LNCaP) with human stromal cells.
- Identification of morphological and growth changes in LNCaP cells in response to human prostate stromal TGF-β signaling.
- Optimization of culture conditions to label human prostate stromal cells with "CellTracker Red CMTPX" from Molecular Probes™
- Testing the quantification methods to assess LNCaP cell proliferation in the LNCaP-stromal cell co-cultures.

Reportable Outcomes:

During the initial year, we published a manuscript entitled "Fibroblast growth factor-2 mediates transforming growth factor-beta action in prostate cancer reactive stroma" in Oncogene (15). This work is in direct relevance to this project. In this manuscript, we show that attenuation of TGF-β signaling by either knockout of TβRII or overexpression of dominant negative Smad3 in mouse prostate stromal cells results in an inhibition of angiogenesis and tumor growth in a human prostate cancer xenograft model. Furthermore, we demonstrated that TGF-β both up-regulates the expression of FGF-2 and promotes the secretion of 18 kDa isoform of FGF-2. Finally, re-expression of FGF-2 in stromal cells with inhibited TGF-β signaling restores angiogenesis and tumor growth *in vivo*. These data fully supports the present project since it was our original hypothesis that the inhibition of TGF-β signaling in prostate stromal cells by SD-208 will attenuate prostate cancer progression, partially due to a remodeling of prostate cancer associated stromal microenvironment.

Conclusions:

In this study, we propose to address the role of SD-208, a TβRI specific inhibitor, in modifying prostate carcinoma associated reactive stroma biology and its effects on prostate cancer by using both *in vitro* co-culture model and *in vivo* xenograft model.

We encountered a problem in obtaining sufficient amount of SD-208 inhibitor at the beginning of the project due to an unexpected closure of *Scios Inc*. Based on the limited amount of SD-208 compound available to us, we have modified our original *Specific Aims* and got the approval from

DOD PCRP for carrying out experiments under these revised **Tasks**.

In this initial grant period, we have demonstrated the SD-208 efficiency in inhibiting TGF-β signaling in human prostate stromal cells *in vitro*. We also optimized various experimental conditions for testing the inhibition of stromal TGF-β signaling by SD-208 on prostate cancer cells in the *in vitro* co-culture model. By using this *in vitro* co-culture model, we have demonstrated that SD-208 changes prostate cancer cell biology by inhibiting TGF-β signaling in prostate stromal cells *in vitro*. Our current cDNA microarray experiments will reveal more details in molecular events in regulating these changes.

Since the *in vivo* xenograft experiments will consume most of our SD-208 stock, we have held these *in vivo* experiments until a demonstration of a SD-208 induced prostate cancer biology in the *in vitro* co-culture experiments. We have now demonstrated such a SD-208 induced biology in our *in vitro* co-culture experiments. Therefore, we are beginning carrying out the *in vivo* xenograft experiments. Since the longest *in vivo* xenograft experiments will be 28 days, we are confident that we will finish all the *in vivo* experiments at the conclusion of the award and present these sets of data in our final report.

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ORIGINAL ARTICLE

Fibroblast growth factor-2 mediates transforming growth factor-β action in prostate cancer reactive stroma

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Transforming growth factor-β (TGF-β) is overexpressed at sites of wound repair and in most adenocarcinomas including prostate cancer. In stromal tissues, TGF-β regulates cell proliferation, phenotype and matrix synthesis. To address mechanisms of TGF-β action in cancer-associated reactive stroma, we developed prostate stromal cells null for TGF-β receptor II (TβRII) or engineered to express a dominant-negative Smad3 to attenuate TGF-β signaling. The differential reactive stroma (DRS) xenograft model was used to evaluate altered stromal TGF-β signaling on LNCaP tumor progression. LNCaP xenograft tumors constructed with TβRII null or dominant-negative Smad3 stromal cells exhibited a significant reduction in mass and microvessel density relative to controls. Additionally, decreased cellular fibroblast growth factor-2 (FGF-2) immunostaining was associated with attenuated TGF-β signaling in stroma. *In vitro*, TGF-β stimulated stromal FGF-2 expression and release. However, stromal cells with attenuated TGF-β signaling were refractory to TGF-β-stimulated FGF-2 expression and release. Re-expression of FGF-2 in these stromal cells in DRS xenografts resulted in restored tumor mass and microvessel density. In summary, these data show that TGF-β signaling in reactive stroma is angiogenic and tumor promoting and that this effect is mediated in part through a TβRII/Smad3-dependent upregulation of FGF-2 expression and release.
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Keywords: transforming growth factor-β; fibroblast growth factor; prostate cancer; stroma; angiogenesis

Introduction

Several studies suggest that a reactive stroma microenvironment affects rate of carcinoma progression, although key factors and signaling mechanisms are poorly understood. Transforming growth factor-β (TGF-β) is overexpressed in most carcinomas and regulates diverse functions of stromal cells through both Smad-dependent and -independent signaling pathways (Coffey *et al*., 1986; Roberts *et al*., 1986; Roberts and Sporn, 1996; Derynck and Zhang, 2003). TGF-β modulates stromal cell phenotype, promotes matrix remodeling, promotes angiogenesis and affects immune responses at sites of tumor formation (Rowley, 2007). Most of these actions are considered tumor promoting. TGF-β has also been considered a tumor suppressor, as it inhibits proliferation of normal epithelial cells; however, many carcinoma cells become refractory to the growth inhibitory activity of TGF-β due to loss or mutation of various TGF-β signaling pathway components in these cells during tumorigenesis (Akhurst and Derynck, 2001). Therefore, understanding the net effects of TGF-β in carcinoma progression is complicated. Net response to TGF-β is likely to be dynamic during evolution of a tumor and will reflect the combined effects of this factor on both carcinoma cells and on all components of the reactive stroma microenvironment.

We have previously shown that a reactive stroma co-evolves early in human prostate cancer during the formation of pre-malignant prostatic intraepithelial neoplasia (PIN) (Tuxhorn *et al*., 2002a). This reactive stroma was typified by carcinoma-associated fibroblasts, myofibroblasts and matrix remodelling. Elevated TGF-β expression was observed in PIN epithelia and prostate cancer cells. We also showed that TGF-β could induce human prostate fibroblasts to a myofibroblast phenotype *in vitro* with elevated expression of tenascin, a marker of reactive stroma (Tuxhorn *et al*., 2002a). Our previous studies have developed a xenograft model (differential reactive stroma, DRS) that recombines LNCaP prostate carcinoma cells with engineered prostate stromal cells (Tuxhorn *et al*., 2002b, c; Yang *et al*., 2005). In these studies we have shown that reactive stroma promotes experimental prostate cancer progression and much of this was due to stromal regulation of angiogenesis (Tuxhorn *et al*., 2002b). Using this same experimental xenograft model, we have also reported that both TGF-β and connective tissue growth factor (CTGF), a downstream mediator of TGF-β action, stimulate angiogenesis and promote tumorigenesis (Tuxhorn *et al*., 2002c; Yang *et al*., 2005). In contrast, under different conditions with different models and carcinoma/stromal cell lines, other studies showed that

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loss of TGF-β signaling in stroma resulted in elevated expression of tumor-promoting factors (TGF-α and HGF) and a net tumor progression (Bhowmick *et al*., 2004; Cheng *et al*., 2005). Together, these studies support the concept that the actions of TGF-β and specific signaling pathways, including downstream mediators expressed in reactive stroma, is an important area for further study. In addition, although the role of Smad3-mediated TGF-β signaling in tissue fibrosis or wounding has been studied (Roberts *et al*., 2003; Lakos *et al*., 2004), it is not known whether Smad3 mediates TGF-β induction of angiogenesis and reactive stroma during carcinoma progression. Accordingly, in order to more clearly define the complicated regulation of carcinogenesis by TGF-β action in stroma, the purposes of this study were to determine whether Smad3-mediated TGF-β signaling is a key pathway in cancer-associated stroma and to subsequently assess candidate downstream mediators of TGF-β/Smad3 biological action.

In this report, we use the LNCaP carcinoma/prostate stromal cell recombined DRS xenograft model to show that loss of TGF-β signaling in prostate stromal cells through either a targeted knockout of TGF-β receptor II (TβRII) or by expression of a dominant-negative Smad3 results in an inhibition of the angiogenesis- and tumor-promoting function of reactive stroma. In addition, we show that the angiogenic and tumorigenic function of stromal TGF-β signaling is mediated, in part, by induced expression and release of FGF-2 from prostate stromal cells in a TβRII/Smad3-dependent manner.

Results

*Attenuated TGF-*β *response in prostate stromal cells*

The TβRII$^{flox/flox}$H, TβRII KO, TβRII CT, Smad3 Ctrl and Smad3 DN prostate stromal cell lines were generated as described in Materials and methods. Table 1 summarizes the designated name for each prostate stromal cell line and how each cell line was derived and/or engineered. In TβRII$^{flox/flox}$H and TβRII CT control cells, TGF-β1 (50 pM) stimulated a seven- to eight-fold expression of p800Luc (PAI-1 promoter), a three-fold expression of α-SMAp-luc (smooth muscle α-actin promoter) and a three- to four-fold expression of pVim-luc (vimentin promoter) (Figure 1a). In contrast, there was no significant TGF-β1-induced promoter activity with any of these constructs in the TβRII KO cells. TGF-β1 also induced a 250- to 270-fold expression of $(CAGA)_{12}$MLP (Smad binding sequence) in control cell lines, whereas this induction was restricted to 2.6-fold in TβRII KO cells (Figure 1a). Our previous study showed that TGF-β1 induces a myofibroblast/smooth muscle phenotype in prostate stromal cells *in vitro* (Tuxhorn *et al*., 2002a). Concordantly, TGF-β1 induced smooth muscle α-actin filament formation in TβRII CT control cells; however, TβRII KO cells were refractory to TGF-β-induced filament formation (Figure 1b).

Table 1 Derivation and engineering of prostate stromal cell lines

Prostate stromal cell line	Method of cell line generation
TβRII$^{flox/flox}$ H	Parent cell line derived from Tgfbr2$^{floxE2/floxE2}$ mouse
TβRII KO[a]	TβRII$^{flox/flox}$ H cells infected with Cre recombinase
TβRII CT[a]	TβRII$^{flox/flox}$ H cells infected with mutant Cre recombinase
C57B	Parent cell line derived from C57BL/6 mouse
Smad3 DN[b]	C57B cells infected with pLPCX-Smad3ΔSSVS
Smad3 Ctrl[b]	C57B cells infected with empty pLPCX vector
TβRII KO + FGF-2[c]	TβRII KO cells infected with pBMN-FGF-2-GFP
TβRII KO + Ctrl[c]	TβRII KO cells infected with empty pBMN-I-eGFP vector
Smad3 DN + FGF-2[d]	Smad3 DN cells infected with pBMN-FGF-2-GFP
Smad3 DN + Ctrl[d]	Smad3 DN cells infected with empty pBMN-I-eGFP vector

[a]Tumor data compared in Figure 3a. [b]Tumor data compared in Figure 3b. [c]Tumor data compared in Figure 6c. [d]Tumor data compared in Figure 6d.

Expression of dominant-negative Smad3 in prostate stromal cells also attenuated TGF-β signaling. Expression of the Flag-Smad3ΔSSVS construct was verified by anti-Flag immunofluorescence (Figure 2a). Attenuation of Smad3 signaling was verified by a significant reduction of TGF-β1-induced expression of the Smad3-responsive $(CAGA)_{12}$MLP reporter in the Smad3 DN cells compared to control (Figure 2b).

*Attenuated stromal TGF-*β *signaling in LNCaP + stroma xenografts*

Construction of LNCaP/prostate stromal cell xenografts and evaluation time points followed protocols identical to what we have published previously (Tuxhorn *et al*., 2002b, c; Yang *et al*., 2005). Xenografts that were constructed with LNCaP plus TβRII KO stromal cells resulted in a 44.1% decrease in microvessel density ($P<0.0001$, $n=72$ fields from 12 xenografts in each group) and a 49.8% decrease in tumor mass compared to LNCaP plus TβRII CT control stromal cells (Figure 3a) ($P=0.0167$, $n=18$) at the 4-week end point. Histopathology of xenografts in all cases revealed clusters of LNCaP cells adjacent to reactive stroma and vessels similar to what we have reported previously for this model. No obvious histological differences were observed in experimental compared to control xenografts. To compare the morphology of current experiments (Figures 7a and b) with subsequent experiments, Figure 7 shows histology for all xenografts in this study. To confirm the histological observations, carcinoma cell to stromal cell ratios were determined in xenografts as described in Materials and methods. No statistically significant differences were observed in carcinoma cell to stromal cell ratios between experimental and control xenografts within each data set (data not shown).

Similar decreases in mass and microvessel density were observed in LNCaP plus Smad3 DN xenografts

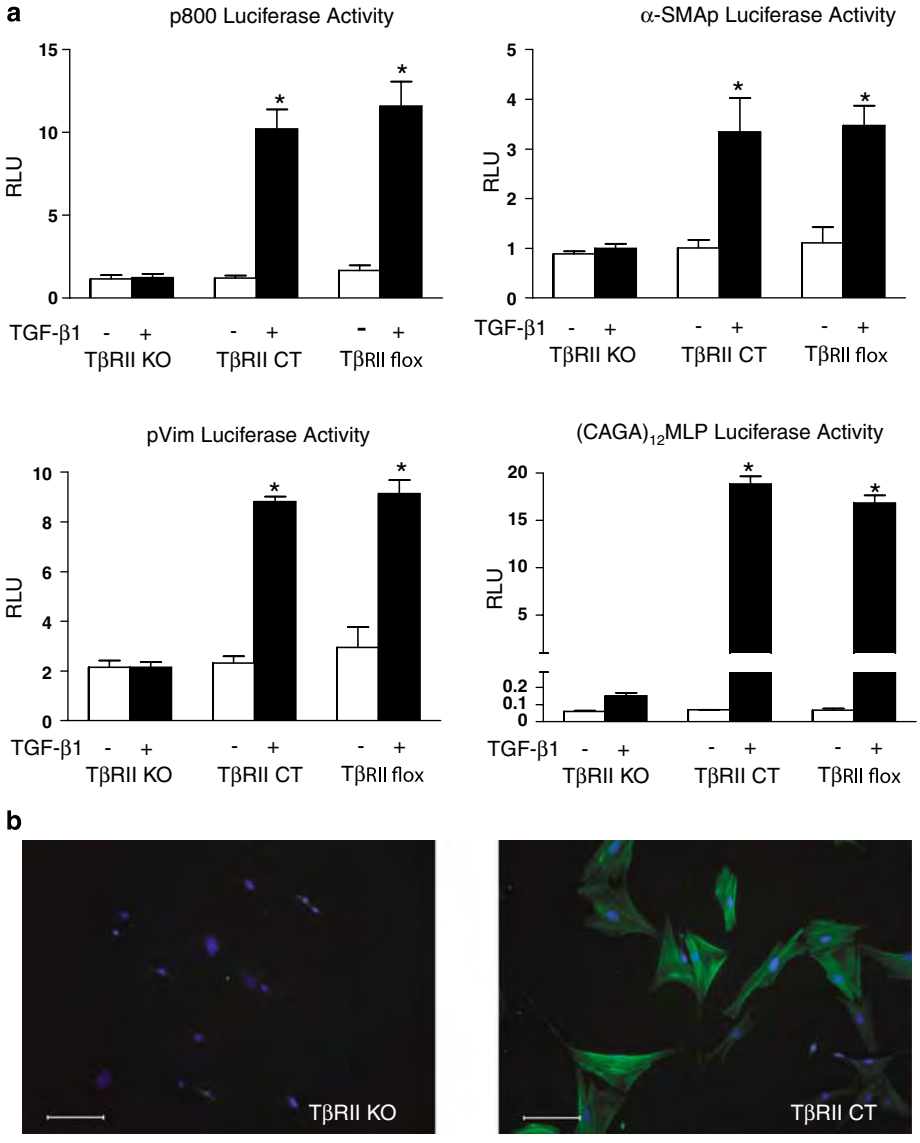

Figure 1 Attenuation of TGF-β signaling in TβRII KO prostate stromal cells. (**a**) TβRII KO, TβRII CT and the parent TβRII$^{flox/flox}$H prostate stromal cells (TβRII flox) were transfected with the TGF-β-responsive luciferase reporters, p800Luc, α-SMAp-luc, pVim-luc or $(CAGA)_{12}$MLP. Cultures were then exposed to either vehicle control (−) or TGF-β1 (50 pM) (+) for 24 h and assayed for luciferase activity, which was normalized to the co-transfected pRL-null-expressed Renilla luciferase activity. Data shown are mean ± s.e.m. derived from replicate-independent experiments. *Significant difference ($P<0.05$). (**b**) In addition, smooth muscle α-actin filament formation was stimulated by TGF-β in TβRII CT, but not TβRII KO cells. TGF-β, transforming growth factor-β; TβRII, TGF-β receptor II.

evaluated at both 2-week (day 14) and 4-week (day 28) end points. LNCaP/Smad3 DN xenografts exhibited a 24.5% decrease in mean mass at 2 weeks compared to control LNCaP/Smad3 Ctrl xenografts ($P=0.0014$, $n=18$) (data not shown). At the 4-week end point, a 31.4% decrease in microvessel density ($P=0.0089$, $n=72$ fields/12 xenografts) and a 40.6% decrease in mean wet weight ($P=0.0216$, $n=18$) was observed in LNCaP/Smad3 DN xenografts relative to LNCaP/Smad3 Ctrl xenografts (Figure 3b). As discussed above, no differences in histology or carcinoma cell to stromal cell ratios were observed (Figures 7d and e).

TGF-β1 induces FGF-2 mRNA and protein expression in stromal cells

Stromal cells were examined by quantitative real-time PCR (qPCR) to assess whether TGF-β1 stimulated FGF-2 mRNA expression. TGF-β1 stimulated expression of FGF-2 mRNA by 2- to 7-fold in both the TβRII CT ($P=0.0332$) and Smad3 Ctrl control ($P=0.0133$) prostate stromal cells (Figure 4a). In contrast, FGF-2 mRNA remained at basal levels and was refractory to TGF-β1 stimulation in both the TβRII KO and Smad3 DN prostate stromal cells. Consistent with this observation, stromal FGF-2 immunoreactivity was greatly decreased in LNCaP/TβRII KO xenografts

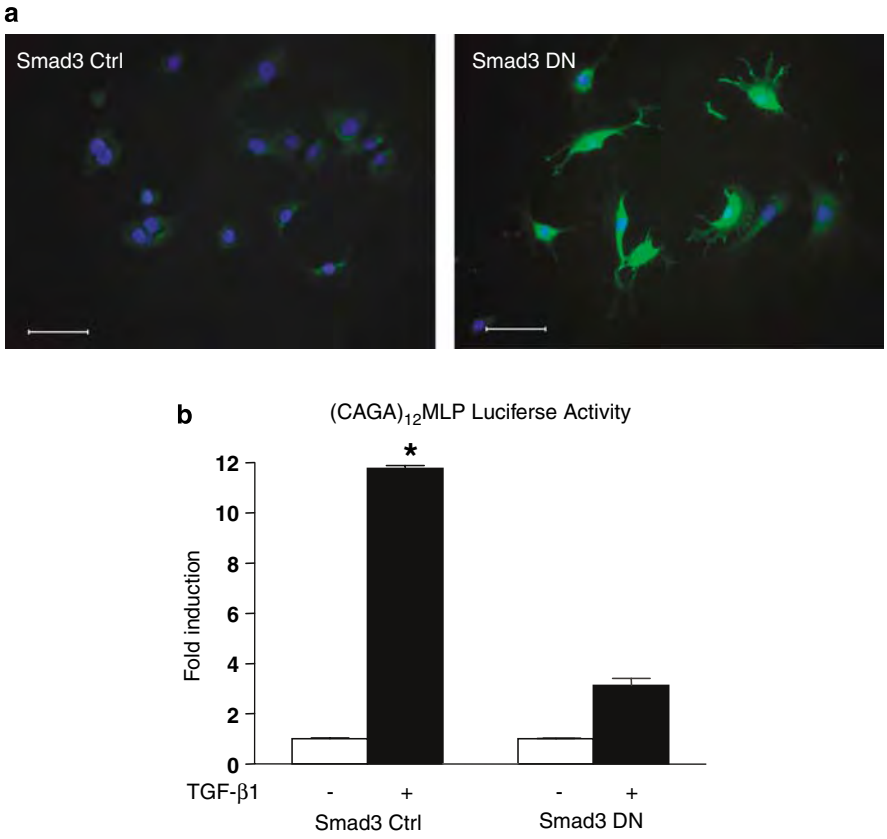

Figure 2 Attenuation of TGF-β signaling in C57B-Smad3 DN prostate stromal cells. (**a**) Flag-Smad3ΔSSVS expression was verified by immunofluorescence using an anti-Flag antibody. (**b**) Smad3 Ctrl and Smad3 DN prostate stromal cell lines were subsequently transfected with the Smad3-responsive luciferase reporter, $(CAGA)_{12}$MLP, and treated with TGF-β. Smad3 DN expression significantly attenuated $(CAGA)_{12}$MLP responsiveness to TGF-β stimulation. TGF-β, transforming growth factor-β.

compared to control LNCaP/TβRII CT xenografts (Figure 4b).

TGF-β1 induces FGF-2 release in stromal cells

Extracts from stromal cells stimulated with TGF-β1 showed that total cellular FGF-2 protein was significantly elevated in both TβRII CT and Smad3 Ctrl cells as determined by enzyme-linked immunosorbent assay (ELISA) ($P=0.0166$ and $P<0.0001$, respectively; Figure 5a). This effect was abrogated in TβRII KO and Smad3 DN cells. Detection of FGF-2 released into conditioned media by control cells was below the ELISA detection threshold. Hence, to determine whether TGF-β1 also induces FGF-2 protein secretion/release, TβRII CT, TβRII KO, Smad3 Ctrl and Smad3 DN prostate stromal cells were each engineered to overexpress an 18 kDa FGF-2-GFP (green fluorescent protein) fusion protein. Under these conditions, TGF-β1 induced a significant, dose-dependent secretion/release of FGF-2 protein into the conditioned media in both the control TβRII CT + FGF-2 ($P=0.0472$) and the Smad3 Ctrl + FGF-2 cells ($P=0.0085$) (Figure 5b). Conversely, TGF-β1 had no effect on FGF-2 release from the TβRII KO + FGF-2 or Smad3 DN + FGF-2 cells. As a control, western blot results show that ectopic FGF-2 was expressed at equivalent levels in control cells and cells with attenuated TGF-β signaling (Figure 5b, inset).

Tβ RII/Smad3-mediated FGF-2 expression in prostate stromal cells promotes angiogenesis and xenograft tumor growth

To determine whether the tumor-inhibiting effects of attenuated TGF-β signaling in stromal cells could be attributed to decreased FGF-2 expression, xenografts were constructed with LNCaP cells plus either TβRII KO or Smad3 DN cells engineered to overexpress the 18 kDa FGF-2-GFP fusion protein (TβRII KO + FGF-2 and Smad3 DN + FGF-2, respectively) or the empty vector control (TβRII KO + Ctrl or Smad3 DN + Ctrl) as described above. LNCaP/TβRII KO + FGF2 xenografts exhibited a recovery of stromal FGF-2 immunostaining (Figure 6a) and this was due to the expression of the FGF-2-GFP fusion protein as these cells were also positive for GFP (Figure 6b). Overexpression of FGF-2 under these conditions produced a 40.0% increase in xenograft mass in LNCaP/TβRII KO + FGF2 xenografts compared to LNCaP/TβRII KO + Ctrl xenografts at the 2-week end point ($P=0.0127$, $n=18$) (data not shown). This expanded to a 141.1% increase in xenograft mass in LNCaP/TβRII KO +

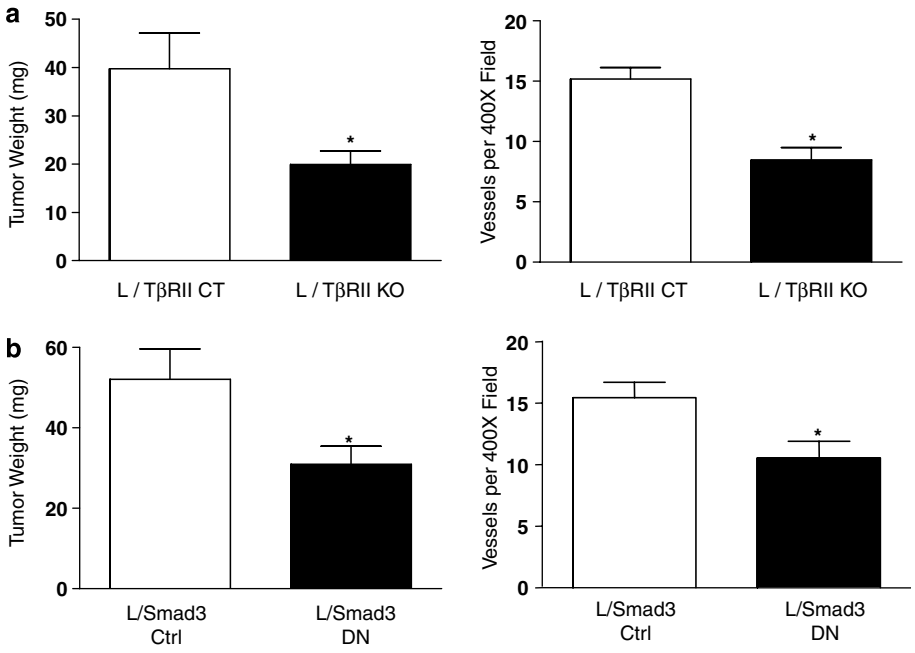

Figure 3 Attenuation of TGF-β signaling in stromal cells inhibits angiogenesis and tumor growth in LNCaP xenografts. (**a**) A significant decrease in xenograft mass was observed at day 28 in LNCaP/TβRII KO xenografts relative to control LNCaP/TβRII CT xenografts. LNCaP/TβRII KO xenografts also exhibited a significant decrease in the mean microvessel density compared to control LNCaP/TβRII CT xenografts. (**b**) Similarly, a significant decrease in xenograft weight was observed at day 28 in LNCaP/Smad3 DN xenografts relative to control LNCaP/Smad3 Ctrl xenografts. Concordantly, LNCaP/Smad3 DN xenografts exhibited a significant decrease in mean microvessel density compared to control LNCaP/Smad3 Ctrl xenografts. *Significant difference ($P<0.05$). TGF-β, transforming growth factor-β; TβRII, TGF-β receptor II.

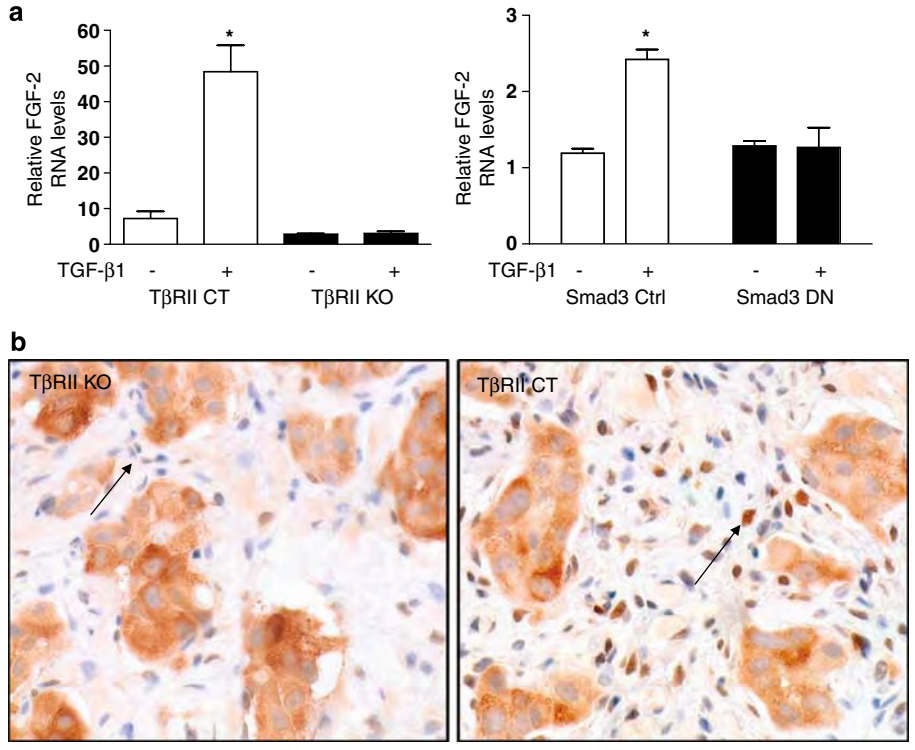

Figure 4 TGF-β1 induces FGF-2 expression in prostate stroma. (**a**) qPCR data showed a significant upregulation of FGF-2 mRNA in TGF-β1-treated TβRII CT or Smad3 Ctrl cells, but not in TβRII KO or Smad3 DN cells. (**b**) Immunohistochemistry revealed positive FGF-2 staining in TβRII CT cells, but little to no immunoreactivity was observed in TβRII KO stromal cells in LNCaP/Stroma xenografts ($\times 400$). Clusters of immunoreactive cells are LNCaP epithelia and arrows point to stromal cells surrounding epithelia. TGF-β, transforming growth factor-β; FGF-2, fibroblast growth factor-2; qPCR, quantitative PCR; TβRII, TGF-β receptor II.

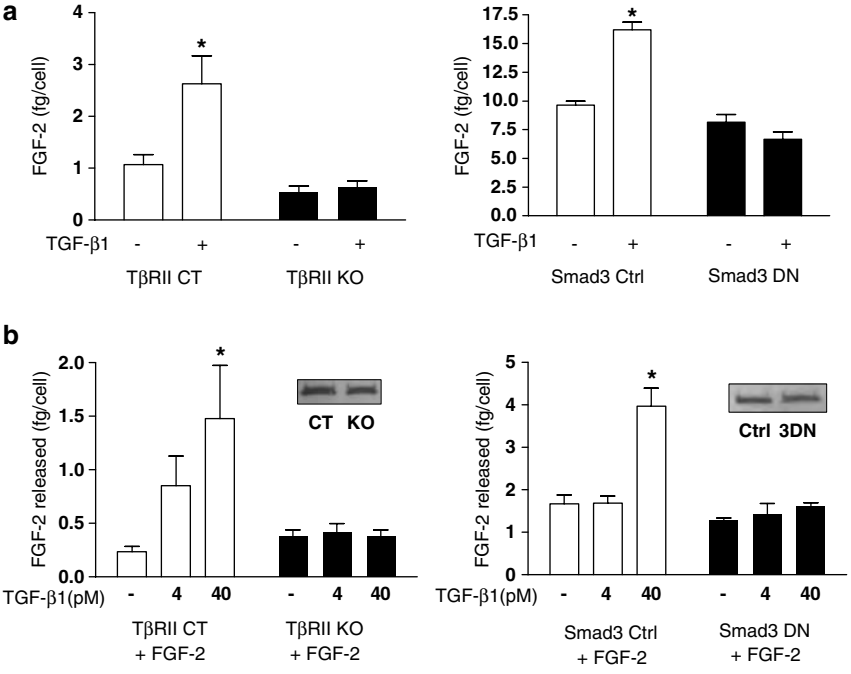

Figure 5 TGF-β1 induces FGF-2 protein expression and secretion/release in prostate stroma. (**a**) ELISA results showed a significant increase in FGF-2 protein levels in TGF-β1-treated TβRII CT and Smad3 Ctrl cells, but not in TβRII KO or Smad3 DN cells. (**b**) TβRII CT, TβRII KO cells, Smad3 Ctrl and Smad3 DN cells were infected to express equivalent levels of FGF-2-GFP protein, as shown by western blot (anti-GFP, inset). ELISA results revealed a significant increase in FGF-2 protein released from TGF-β1-treated TβRII CT + FGF-2 and Smad3 Ctrl + FGF-2 cells in a dose-dependent manner, whereas this response was inhibited in either TβRII KO + FGF-2 or Smad3 DN + FGF-2 cells. *Significant difference ($P<0.05$). TGF-β, transforming growth factor-β; FGF-2, fibroblast growth factor-2; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; TβRII, TGF-β receptor II.

FGF2 xenografts compared to LNCaP/TβRII KO + Ctrl xenografts at the 4-week end point ($P<0.0001$, $n=18$). In addition, the average xenograft mass of LNCaP/TβRII KO + FGF-2 xenografts (44.14 ± 5.82 mg) was comparable to the control LNCaP/TβRIICT tumor mass of 39.77 ± 7.36 mg at 4 weeks with native FGF-2 expression with intact TGF-β signaling. Concordantly, LNCaP/TβRII KO + FGF2 xenografts showed a 38.9% increase in microvessel density compared to control LNCaP/TβRII KO + Ctrl xenografts ($P=0.0307$, $n=72$ fields/12 xenografts) (Figure 6c). Similarly, overexpression of FGF-2 in Smad3 DN stromal cells produced a 30.4% increase in LNCaP/Smad3 DN + FGF-2 xenograft mass compared to LNCaP/Smad3 DN + Ctrl control xenografts at the 2-week end point ($P=0.0138$, $n=18$; Figure 6d). Interestingly, the mean mass in LNCaP/Smad3 DN + FGF-2 xenografts (24.15 ± 1.76 mg) is comparable to the mean mass of 2-week control LNCaP/Smad3 Ctrl xenografts (28.46 ± 1.21 mg) with intact Smad3 signaling. Furthermore, LNCaP/Smad3 DN + FGF-2 xenografts showed a 45.3% increase in microvessel density compared to control LNCaP/Smad3 DN + Ctrl xenografts ($P=0.0027$, $n=72$ fields/12 xenografts; Figure 6d). Histopathologic analysis showed that there were no obvious differences in histology between control xenografts and xenografts with attenuated TGF-β signaling or xenografts with attenuated TGF-β signaling and overexpression of FGF-2 (Figure 7). As in previous experiments, there were no statistically significant differences in carcinoma to stromal cell ratios in these experiments (data not shown).

Discussion

Data presented here represent the first direct experimental evidence that links TGF-β signaling in the stromal compartment with the angiogenic, tumor-promoting effects of reactive stroma in an experimental human prostate cancer model. These data show that tumor-promoting TGF-β signaling in prostate stromal cells is mediated through a Smad3 pathway, although our results do not rule out other TGF-β-activated signaling pathways. In addition, these data show that the biological action of TGF-β signaling in reactive stroma is mediated, in part, through stimulated expression and secretion/release of FGF-2, also in a Smad3-regulated manner. Coordinate TGF-β/FGF-2 signaling in stroma resulted in elevated vessel density and prostate cancer xenograft growth, consistent with pro-reactive stroma and pro-angiogenic activities of both TGF-β1 (Roberts *et al*., 1986) and FGF-2 (Dow and deVere White, 2000). These results suggest that TGF-β1 is a key upstream factor that regulates stromal microenvironment biology during prostate cancer progression and that signaling through Smad3 is a critical pathway. Data

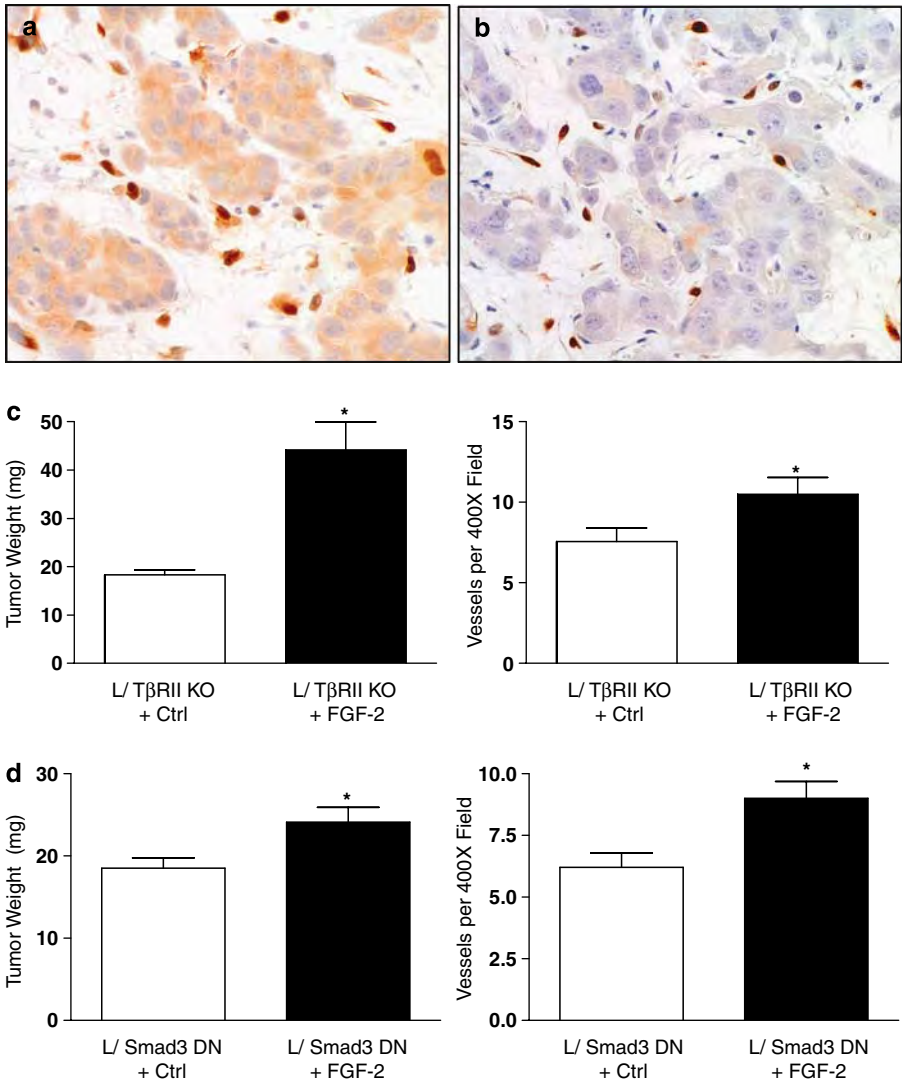

Figure 6 Ectopic expression of FGF-2 in stromal cells with attenuated TGF-β signaling restores LNCaP/Stroma xenograft growth and angiogenesis. Expression of an FGF-2-GFP fusion protein in TβRII KO prostate stromal cells in LNCaP/Stroma xenografts was verified by immunohistochemistry with (**a**) anti-FGF-2 and (**b**) anti-GFP antibodies ($\times 400$). LNCaP/Stroma xenografts constructed with TβRII KO (**c**) or Smad3 DN (**d**) cells ectopically expressing FGF-2 display a significant increase in tumor mass compared to control xenografts. Concordantly, microvessel density was significantly elevated in TβRII KO + FGF-2 and Smad3 DN + FGF-2 xenografts compared to TβRII KO + Ctrl or Smad3 DN + Ctrl xenografts. *Significant difference ($P<0.05$). TGF-β, transforming growth factor-β; FGF-2, fibroblast growth factor-2; GFP, green fluorescent protein; TβRII, TGF-β receptor II.

presented here are consistent with the implication of Smad3 in mediating wound healing and fibrosis. As might be predicted, Smad3 null mice were refractory to TGF-β1-induced pulmonary fibrosis (Bonniaud *et al*., 2004). However, the function of Smad3 in mediating TGF-β-induced responses in cancer-associated reactive stroma biology is poorly understood. Data here suggest that Smad3-mediated pathways may be common to tissue wound repair, fibrosis and reactive stroma in cancer.

Our previous reports have shown that TGF-β is overexpressed in pre-malignant PIN epithelia and that a reactive stroma phenotype co-evolved at sites adjacent to PIN (Tuxhorn *et al*., 2002a). This initial reactive stroma consisted of carcinoma-associated fibroblasts, myofibroblasts and matrix remodeling, typical of a wound repair stroma. Matrix remodeling was typified by overexpression of collagen I, tenascin and fibroblast activation protein. TGF-β1 stimulates synthesis of matrix components by fibroblasts, including collagen type I and promotes angiogenesis in wound repair granulation tissue (Roberts *et al*., 1986; Roberts and Sporn, 1996). In prostate stromal cells, TGF-β1 induced synthesis of collagen type I (Fukabori *et al*., 1997), fibronectin (Butter *et al*., 2001), tenascin (Tuxhorn *et al*., 2002c), versican (Sakko *et al*., 2001) and induced myodifferentiation (Peehl and Sellers, 1998; Tuxhorn *et al*., 2002a). TGF-β1 is overexpressed in many carcinomas, including prostate cancer epithelial cells (Eastham *et al*., 1995), and has been reported as a primary inducer of myofibroblast differentiation in reactive stroma fibroblasts (Desmouliere *et al*., 1993;

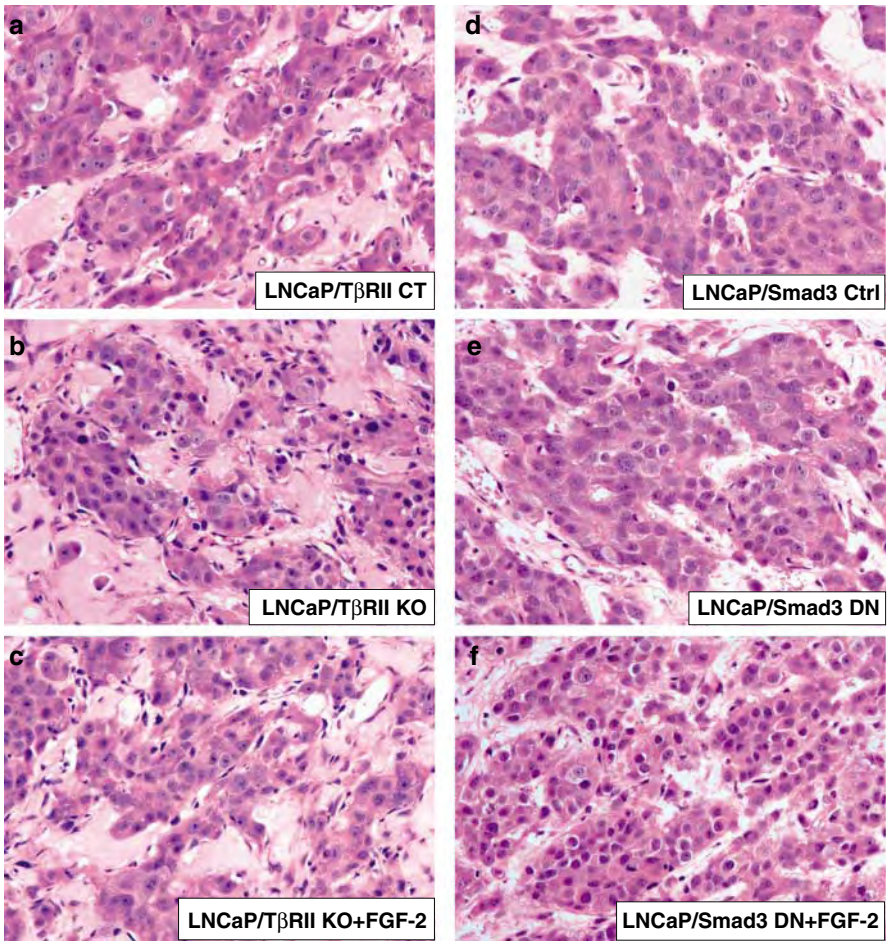

Figure 7 Comparative histology of xenografts. Representative H&E histology (× 400) is shown for each of the following xenografts: (**a**) LNCaP/T*β*RII CT, (**b**) LNCaP/T*β*RII KO, (**c**) LNCaP/T*β*RII KO + FGF-2, (**d**) LNCaP/Smad3 Ctrl, (**e**) LNCaP/Smad3 DN and (**f**) LNCaP/Smad3 DN + FGF-2. H&E, hematoxylin and eosin; T*β*RII, TGF-*β* receptor II.

Tuxhorn *et al*., 2002a). Consistent with this, the myofibroblast is a common stromal cell type in carcinoma-associated stroma in many different epithelial cancers (Ronnov-Jessen *et al*., 1996; Tuxhorn *et al*., 2002a).

The carcinoma-associated reactive stroma in human cancer has been characterized as being similar to a wound repair granulation tissue, which commonly has a high myofibroblast population (Dvorak, 1986; Ronnov-Jessen *et al*., 1996; Rowley, 2007). In granulation tissue, elevated angiogenesis is coordinate with elevated FGF-2 and it is well established that TGF-*β* promotes granulation tissue formation and angiogenesis (Roberts *et al*., 1986). FGF-2 is TGF-*β*-regulated and a mitogen for epithelial and stromal cells while also playing a role in the migration of endothelial cells during blood vessel formation (Dow and deVere White, 2000). Overexpression of FGF-2 in stromal cells is nearly universal in wound repair, fibroses and cancer-associated reactive stroma (Rowley, 2007). In addition to its fibrogenic role during wound repair, a significant upregulation of FGF-2 is seen in a variety of cancers including prostate cancer (Giri *et al*., 1999; Dow and deVere White, 2000). Concordantly, tumor growth in the TRAMP mouse prostate cancer model was slowed in an FGF-2 knockout background (Polnaszek *et al*., 2003). In addition, FGF receptor 1 (FGFR1), a cognate receptor for FGF-2, is upregulated in epithelia during prostate cancer progression in human cancer (Giri *et al*., 1999) and in mouse prostate cancer models (Huss *et al*., 2003). Elevated FGF-2 expression in the stroma might be expected to provide a growth advantage for carcinoma cells with elevated expression of FGFR1. Therefore, it is likely that a TGF-*β*1-regulated and Smad3-mediated expression and release of FGF-2 in reactive stroma provides a pro-angiogenic and pro-tumorigenic microenvironment. Accordingly, the TGF-*β*/FGF-2 signaling axis is likely to be a key regulatory component of carcinoma cell–stromal cell interaction.

Our results do not rule out the involvement of other TGF-*β*-regulated growth factors. We believe that it is highly unlikely that FGF-2 is the only pathway downstream of TGF-*β* in stroma promoting LNCaP tumor growth, but that it does partially mediate the angiogenic action of TGF-*β*. In addition to FGF-2, TGF-*β* stimulates expression of many other growth factors in stromal cells including vascular endothelial growth factor, heparin-binding-epidermal growth factor, interleukin-6

and CTGF (Igarashi *et al*., 1993; Pertovaara *et al*., 1994; Story *et al*., 1996; Uchiyama-Tanaka *et al*., 2002; Hayashi *et al*., 2004; Yang *et al*., 2005), some of which, similar to FGF2, are Smad3 regulated. Hence, focal overexpression of TGF-β at sites of early cancer might be expected to initiate a local reactive stroma, typified by matrix remodeling, induction of myofibroblasts, collagen deposition and induced angiogenesis, similar to the initiation of these events at sites of wound repair, where platelets release TGF-β. It is also becoming more clear that the net response to elevated TGF-β in cancer is likely to be mediated through a diverse set of downstream factors. Accordingly, the biology induced in the tumor microenvironment may be either tumor-promoting or tumor-inhibiting depending on many local variables. Regardless, co-evolution of a reactive stroma does seem to correlate with tumor progression. Hence, it has been suggested by several that stimulation of a local host reactive microenvironment is a key step during transition of pre-neoplastic foci to overt neoplasia (Ronnov-Jessen *et al*., 1996; Liotta and Kohn, 2001; Tuxhorn *et al*., 2001). In this study, we propose that the TGF-β/FGF-2 signaling axis is likely to be one of many coordinate sets of factors that regulate the complex biology of a reactive stroma microenvironment in carcinomas.

Materials and methods

Cell lines
LNCaP and Phoenix E cells were purchased from ATCC (Manassas, VA, USA) and maintained as described previously (Yang *et al*., 2005). The C57B mouse prostate stromal cell line was generated and cultured as reported previously (Yang *et al*., 2005). A prostate stromal cell line was initiated from the ventral prostate of an 8-week Tgfbr2$^{floxE2/floxE2}$ mouse (Bhowmick *et al*., 2004) carrying loxP sites at introns 1 and 2 of the TβRII gene, following the same procedure as reported previously (Yang *et al*., 2005) and named the TβRII$^{flox/flox}$H cells. Passages 8–15 were used for all experiments.

Knockout of TβRII alleles in TβRII$^{flox/flox}$H cells
The HR-MMPCreGFP retroviral construct carrying an bioactive Cre-GFP fusion protein with a lox 511 in the 3′ LTR U3 region or the control HR-MMPCreGFPY324F carrying a loss-of-function CreY324F-GFP mutant (Silver and Livingston, 2001) were transfected into Phoenix E cells with a Calcium Phosphate Transfection kit (Invitrogen, Carlsbad, CA, USA). Virus was collected, filtered (0.45 μm) and applied to infect TβRII$^{flox/flox}$H cells as described previously (Yang *et al*., 2005). Cre-GFP expression in TβRII$^{flox/flox}$H cells excised the floxed *TβRII* alleles and self-excised the lox 511 flanked Cre-GFP (thus 'Hit and Run'), resulting in *TβRII* null cells named TβRII KO. CreY324F-GFP did not excise *TβRII* alleles and the resulting cells were named TβRII CT.

N-Flag-Smad3ΔSSVS expression in C57B prostate stromal cells
To inhibit Smad3-mediated signaling, either pLPCX-N-Flag-Smad3ΔSSVS or control pLPCX (Choy *et al*., 2000) was transfected into Phoenix E cells. Virus was collected, filtered and applied to infect C57B prostate stromal cells. Cells were selected with 2 μg/ml puromycin. The resulting cell lines were named Smad3 DN and Smad3 Ctrl.

Overexpression of FGF-2
FGF-2-GFP cDNA encoding an 18 kDa isoform FGF-2 fused to GFP protein was subcloned from pRev-TRE2 (Backhaus *et al*., 2004) into pBMN-LacZ. Functionality of this protein has been described previously (Backhaus *et al*., 2004). pBMN-FGF-2-GFP or pBMN-I-eGFP control vector was transfected into Phoenix E cells. Virus was collected, filtered and applied to infect TβRII KO or Smad3 DN cells. The resulting cell lines were named TβRII KO + FGF-2, TβRII KO + Ctrl, Smad3 DN + FGF2 and Smad3 DN + Ctrl. Methods for promoter assays, immunocytochemistry, quantitative PCR, ELISA and western blots using cell lines are provided in the Supplementary Information.

Animals and DRS xenografts
Athymic NCr-nu/nu male mice, 6–8 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA, USA). All experiments were in compliance with the NIH Guide for the Care and Use of Laboratory Animals according to institutional guidelines of Baylor College of Medicine.

DRS xenografts were generated following procedures published previously (Tuxhorn *et al*., 2002b, c; Yang *et al*., 2005). Briefly, 2×10^6 LNCaP cells with 5×10^5 of either TβRII CT, TβRII KO, Smad Ctrl, Smad3 DN, TβRII KO + Ctrl, TβRII KO + FGF2, Smad3 DN + Ctrl or Smad3 DN + FGF2 cells in matrigel were injected subcutaneously in each rear lateral flank of three mice for a total of six tumors per experimental group per injection preparation. A minimum of three independent experiments was performed for each combination tested ($n = 18$ tumors). Xenograft tumors were evaluated at either day 14 or 28 after inoculation, as these are optimal intermediate and later stage time points as reported previously (Tuxhorn *et al*., 2002b, c; Yang *et al*., 2005). Xenografts were weighed and fixed in 4% paraformaldehyde at 4°C overnight and paraffin embedded. Sections (5 μm) were mounted onto ProbeOn Plus slides (Fisher, Pittsburgh, PA, USA) and either stained with H&E or processed for immunohistochemistry. Methods for immunohistochemistry, microvessel density counts and determination of carcinoma to stromal cell ratios are included in the Supplementary Information.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).